Promotion of Hepatic Preneoplastic Lesions in Male B6C3F$_1$ Mice by Unleaded Gasoline

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In previous studies, unleaded gasoline (UG) vapor was found to be a liver tumor promoter and hepatocarcinogen in female mice, but UG was not a hepatocarcinogen in male mice. However, UG vapor had similar transient mitogenic effects in nonlesioned liver of both male and female mice under the conditions of the cancer bioassay. We used an initiation–promotion protocol to determine whether UG vapor acts as a liver tumor promoter in male mice and to examine proliferative effects that may be critical to tumor development. Twelve-day-old male B6C3F$_1$ mice were injected with *N*-nitrosodiethylamine (DEN; 5 mg/kg, intraperitoneally) or vehicle. Starting at 5–7 weeks of age, mice were exposed by inhalation 6 hr/day, 5 days/week for 16 weeks to 0 or 2046 ppm of PS-6 blend UG. UG treatment caused a significant 2.3-fold increase in the number of macroscopic hepatic masses in DEN-initiated mice, whereas no macroscopic masses were observed in noninitiated mice. Altered hepatic foci (AHF), which were predominantly basophilic in phenotype, were found almost exclusively in DEN-initiated mice. UG treatment significantly increased both the mean volume (threefold) and the volume fraction (twofold) of the AHF without increasing the number of AHF per unit area. UG also induced hepatic pentoxyresorufin-*O*-dealkylase (PROD) activity, a marker of CYP2B, by more than 12-fold over control with or without DEN cotreatment. To study hepatocyte proliferative effects of UG, we treated mice with 5-bromo-2′-deoxyuridine (BrdU) via osmotic pump for 3 days before necropsy and measured hepatocyte BrdU labeling index (LI) in AHF and nonlesioned liver. UG did not significantly affect BrdU LI in nonlesioned liver. However, hepatocyte LI in AHF was about 30% higher in DEN/UG-treated mice relative to mice treated with DEN alone. These data show that UG vapor promotes AHF in male mice and that liver tumor promotion is associated with a selective increase in hepatocyte proliferation in AHF. UG acts as a liver tumor promoter in both male and female mice, and these findings contrast with the lack of hepatocarcinogenicity of UG in male mice in a cancer bioassay. *Key words*: altered hepatic foci, cell proliferation, liver, gasoline, tumor promotion. *Environ Health Perspect* 103:696–700 (1995)

Enormous quantities of unleaded gasoline (UG) vapor are released into the air each year during transfer of UG to consumers. The general public is most commonly exposed to UG in the form of vapors that evaporate during refueling vehicles at service stations (*1*). The health risk of such intermittent, low-dose exposure to UG vapor is difficult to assess. In a cancer bioassay, a relatively high exposure level of UG vapor (2056 ppm, 6 hr/day, 5 days/week) but not lower levels (67 or 292 ppm UG) increased the incidence of hepatocellular tumors in female B6C3F$_1$ mice (*2*). In contrast, the incidence of hepatocellular tumors in male B6C3F$_1$ mice was not increased at any exposure level of UG (*2,3*). This sex-specific hepatocarcinogenic effect of UG in mice has been the subject of several mechanistic studies. Tilbury et al. (*4*) showed that 2056 ppm UG vapor induced hepatocyte proliferation without evidence of hepatotoxicity in both male and female B6C3F$_1$ mice only in the first week of 13 weeks of intermittent exposure. The lack of sustained hepatocyte proliferation and the induction of proliferation in both sexes of mice make mitogenesis an unlikely explanation for the sex-specific induction of liver cancer by UG. Nonetheless, many hepatic mitogens are known mouse liver tumor promoters (*5*), and UG vapor was subsequently shown to be a weak liver tumor promoter in female B6C3F$_1$ mice initiated with *N*-nitrosodiethylnitrosamine (DEN) (*6*). UG, including the specific blend used in the cancer bioassay, has shown little or no genotoxic activity in a variety of assays (*7–9*).

Recently, MacGregor et al. (*10*) reported that 2056 ppm of UG caused a high incidence of uterine atrophy in female mice in the cancer bioassay, suggesting that UG may disrupt the hormonal balance of female mice. We hypothesized that UG might have antiestrogenic effects and that estrogen antagonism might be causally related to liver tumor promotion in female mice (*11*) because estrogens and/or ovarian factors are known to inhibit liver tumor promotion in mice (*12–18*). Indeed, intermittent exposure to 2056 ppm but not to 292 ppm of UG vapor for 16 weeks promoted the growth of preneoplastic lesions and decreased relative uterine weight in DEN-initiated female mice (*11*). UG also antagonized several pharmacological effects of exogenous ethinyl estradiol (*11*). UG by intragastric intubation caused a dose-dependent increase in estrogen metabolic capacity in hepatocytes isolated from female mice (*19*), thus suggesting a mechanism by which UG might antagonize estrogen. Although UG antagonizes pharmacological levels of estrogen, a clear causal relationship between antagonism of endogenous estrogens and liver tumor promotion by UG has not been established.

An assumption of this antiestrogenic hypothesis of UG carcinogenesis is that antagonism of the presumably low estrogen levels in male mice would be inconsequential with regard to liver tumor promotion, thus accounting for the lack of hepatocarcinogenicity of UG in male mice. Moreover, the strong liver-tumor-promoting effect of androgens might be expected to be the predominant hormonal influence in male mice (*12,13,17*). However, given the similar acute mitogenic response of male and female mouse liver to UG vapor and the potential relationship of mitogenicity to tumor promotion, it was of interest to test whether UG acted as a liver tumor promoter in male mice. In the present study, the possible tumor-promoting activity of UG in male mice was tested in the same initiation–promotion model in which liver tumor promotion by UG was demonstrated in female mice (*5,11*). In addition, the cellular basis of such promoting activity was examined by analysis of hepatocellular proliferation in altered hepatic foci (AHF) and surrounding nonlesioned liver.

Materials and Methods

PS-6 blend UG was provided by the American Petroleum Institute and was from the same lot used in the cancer bioassay. Unless otherwise specified, all other chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri).

All experiments were conducted under NIH guidelines for the care and use of laboratory animals and were approved by the CIIT Institutional Animal Care and Use Committee. Male C3H/HeNCrlBR mice and female C57BL/6NCrlBR mice free of common murine pathogens were obtained from Charles River Breeding Labs (Raleigh, North Carolina) and acclimated for 10 days. Mice were housed individually in polystyrene cages on α-cellulose bed-

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ding in a temperature- and humidity-controlled room. Mice were kept on a 12-hr light–dark cycle, with the light period extending from 0600 hr to 1800 hr. Food (NIH-07 open formula diet; Ziegler Brothers, Gardners, Pennsylvania) and filter-purified tap water were provided *ad libitum*. The mice were then bred, and the resulting $B6C3F_1$ offspring were treated as described below.

At exactly 12 days of age, we injected male $B6C3F_1$ mice intraperitoneally with either 5.0 mg/kg DEN in 0.9% NaCl or 0.9% NaCl alone (7.1 ml/kg). The mice were weaned at 4–6 weeks of age and housed individually as described above.

At 5–7 weeks of age, the $B6C3F_1$ mice from the DEN initiation and NaCl control groups were separately randomized by weight, assigned to one of two groups (n = 12), and transferred to individual hanging stainless-steel cages contained in a 1-m^3 whole-body inhalation chamber. The mice were exposed to 0 or 2046 ppm (target concentration) of wholly vaporized PS-6 blend UG for 6 hr/day, 5 days/week, for 16 weeks. Exposures were routinely conducted from 0800 hr to 1400 hr on weekdays, including holidays. The chamber design, exposure generation system, and monitoring system were exactly as described previously (*5*), and chamber concentrations of UG were determined hourly. Average daily chamber concentrations of UG ranged from 1741 to 2146 ppm, with a mean and SD of 2046 ± 63 ppm (99.5% of target level). Filter-purified tap water was available *ad libitum*, whereas food was only available during nonexposure periods. We recorded clinical observations and body weights weekly.

Three days before necropsy, mice were implanted subcutaneously with osmotic pumps (Alzet model 2001, 1 µL/hr; Alza Corporation, Palo Alto, California) containing 16 mg/mL 5-bromo-2′-deoxyuridine (BrdU) dissolved in phosphate-buffered saline (Gibco-BRL, Bethesda, Maryland). The pH of the BrdU solution was adjusted to 7.2 ± 0.2 with sodium hydroxide.

We evaluated BrdU incorporation immunohistochemically in liver sections and hepatocyte labeling index (LI) in nonlesioned areas of liver sections as described previously (*5*). At least 2000 nuclei in the left lobe were counted with the experimenter blind to the treatment group.

For preneoplastic lesions, all hepatocellular nuclei of all basophilic lesions that completely fit within the grid of a 10× objective were counted. The maximum area of such a lesion was calculated to be 0.58 mm^2, which corresponds to a focal diameter of 0.85 mm. We chose this cutoff point because of the technical difficulty of determining BrdU labeling indices of larger lesions. The preneoplastic lesions were readily identifiable in hematoxylin and eosin (H&E)-stained sections and were further delineated by their high labeling indices relative to nonlesioned liver.

Approximately 20 hr after the last inhalation exposure, mice were weighed, anesthetized with isoflurane, and exsanguinated. Blood collected by cardiac puncture was allowed to clot for 0.5–1.0 hr and then centrifuged to obtain serum. Testes were removed and weighed together. Livers were removed, weighed, and examined for the presence of macroscopic lesions. The number of macroscopic hepatic masses ≥1 mm was determined. Sections of the left, median right, and right anterior lobes were fixed in 10% buffered formalin. The balance of the liver was minced, rinsed with ice-cold isotonic KCl-Tris (0.154 M KCl, 0.050 M Tris, pH 7.4), chilled on ice, and used to prepare microsomes as described previously (*5*). Microsomes were pooled from three mice in the same treatment group.

The formalin was replaced by 70% ethanol 48 hr after necropsy. Tissues were embedded in paraffin, sectioned at 5 µm, stained with H&E, and examined microscopically. Pituitaries were removed after fixation in formalin, weighed to the nearest milligram, and processed for microscopic examination.

We determined the total area of liver at each sample site occupied on an H&E-stained section from the inhalation experiment using an Image-1 image processing system (Universal Imaging Corporation, West Chester, Pennsylvania). Sections were examined for the presence of AHF ≥10 cells in size with the experimenter blind to the treatment group, and foci were classified according to histopathological phenotype using standard criteria (*20*). We recorded the area of each focus and used it to calculate the number and volume of foci according to the stereological method of Pugh et al. (*21*) using a focal profile cutoff with a radius of 65 µm.

We assayed hepatic microsomal pentoxyresorufin-*O*-dealkylase (PROD) activity essentially as described by Lubet et al. (*22*). Microsomal protein was assayed with Coomassie-plus protein assay reagent (Pierce, Rockford, Illinois) using bovine serum albumin (Pierce) as a standard. Sorbitol dehydrogenase (SDH) activity in serum was determined immediately after necropsy using a commercial kit (#50-UV) from Sigma.

Table 1. Final body weights, relative liver weight, hepatic PROD activity, and serum SDH activity in male $B6C3F_1$ mice

Treatment[a]	Final body weight[b] (g)	Liver weight (% of body weight)[b]	Hepatic PROD activity[c] (pmol/min/mg)	Serum SDH activity[b] (U/L)
Saline/control	35.2 ± 2.3	5.03 ± 0.36	18.5 ± 2.9	24.5 ± 4.0
Saline/UG	34.7 ± 2.1	5.82 ± 0.81*	215.0 ± 19.9*	30.8 ± 6.0
DEN/control	35.6 ± 2.4	6.36 ± 0.56**	11.2 ± 2.2	27.3 ± 4.1
DEN/UG	36.0 ± 2.8	8.30 ± 0.86**	172.0 ± 27.4**	35.0 ± 8.7

Abbreviations: PROD, pentoxyresorufin-*O*-dealkylase; SDH, sorbital dehydrogenase; DEN, *N*-nitrodiethylamine; UG, unleaded gasoline.
[a]Male $B6C3F_1$ mice were injected with DEN (5 mg/kg, intraperitoneally) or vehicle (saline) at 12 days of age. Beginning at 5–7 weeks of age, mice were treated with 0 (control) or 2046 ppm UG vapor for 6 hr/day and 5 days/week for 16 weeks.
[b]Values are the means ± SD of 11–12 mice.
[c]Values are the means ± SD of four microsomal samples that were each pooled from three mice.
*$p < 0.05$ as compared to saline/control group.
**$p < 0.05$ as compared to DEN/control group.

Table 2. Number of gross hepatic masses and parameters of altered hepatic foci in DEN-initiated mice

Promotion treatment[a]	No. of gross hepatic masses ≥1 mm	Altered hepatic foci: Density (No./liver)[b]	Altered hepatic foci: Mean volume (103 × mm^3)	Altered hepatic foci: Volume fraction (%)
Saline/control	0	0[c]	0	0
Saline/UG	0	0[c]	0	0
DEN/control[d]	23.3 ± 13.6	802 ± 419	292.7 ± 210.0	9.51 ± 5.72
DEN/UG[d]	52.9 ± 20.1*	725 ± 259	851.9 ± 512.1*	17.75 ± 8.16*

Abbreviations: DEN, *N*-nitrosodiethylamine; UG, unleaded gasoline.
[a]Male $B6C3F_1$ mice were injected with DEN (5 mg/kg, intraperitoneally) at 12 days of age. Beginning at 5–7 weeks of age, mice were treated with 0 (control) or 2046 ppm UG vapor for 6 hr/day and 5 days/week for 16 weeks.
[b]Assumes 1 g liver = 1 cm^3.
[c]Only one altered hepatic focus was detected in one animal of each of these groups. Thus, calculation of focal parameters is omitted for these groups.
[d]Values are means ± SD of 11–12 mice.
*$p < 0.05$ as compared to DEN control.

We compared mean values for control and DEN-initiated mice to the corresponding UG-treated group by an unpaired, two-tailed *t*-test. To fulfill the requirement for homogeneity of variance, foci data were log transformed before statistical analysis. Differences were considered significant at $p < 0.05$.

Results

Inhalation exposures were not begun until the mice were 5–7 weeks old because that is the age at which exposures were begun in the cancer bioassay of UG. Treatment with DEN, UG, or the combination did not significantly affect body weight relative to controls at any time point (data not shown), and no adverse clinical signs were observed. One DEN/UG-treated mouse died as a result of a cage accident.

Treatment with UG increased liver weight with respect to controls for both noninitiated and DEN-initiated mice (Table 1). UG-induced hepatomegaly was greater in DEN-initiated mice, evidently due to the presence of numerous pale, white lesions in that group. DEN-initiated mice not exposed to UG had approximately half as many macroscopic neoplasms as in the DEN/UG group, and no neoplasms were observed grossly in initiation control mice (Table 2). Testicular weight and pituitary weight were not significantly affected by UG exposure (data not shown).

Induction of hepatic PROD activity, which is catalyzed principally by CYP2B (*23,24*), has been correlated with liver tumor promotion in rodents (*25,26*). Because hepatic PROD activity was induced in female mice exposed to 2056 ppm UG for 13 weeks (*5*), the possible effect of UG on hepatic PROD activity in male mice was determined. UG induced hepatic PROD by about 12-fold in uninitiated mice and by about 16-fold in DEN-initiated mice relative to corresponding controls (Table 1).

Serum SDH activity, a marker of hepatic necrosis, was not significantly elevated by UG treatment (Table 1), which is consistent with the lack of hepatotoxicity in male or female mice exposed to up to 2056 ppm UG for 13 weeks (*4*).

Testes and pituitaries of DEN, UG, and DEN/UG-treated mice were histologically indistinguishable from those of control mice. Modest centrilobular hepatocyte swelling was observed in H&E-stained liver sections from mice treated with 2056 ppm UG with or without DEN treatment; however, no hepatic necrosis was observed. In DEN-initiated mice, a large number of AHF were observed in H&E liver sections. Only one AHF was found in one animal in each of the noninitiated groups. Approximately 90% of the AHF were basophilic, and the majority of the remainder were mixed (basophilic/clear cell). Because the vast majority of AHF were basophilic, AHF were grouped together for the purpose of stereological analysis. As shown in Table 2, UG treatment increased the mean volume and volume fraction occupied by AHF by threefold and twofold, respectively. While this may seem to be a subtle effect, it is worth noting that the AHF in the DEN/control group were clearly at an advanced stage of development as judged by the relatively large mean volume and volume fraction occupied by AHF (Table 2). Thus, a twofold increase in volume fraction when volume fraction was already near 10% in the DEN/control group represents a highly biologically significant increase. The number of AHF per unit area, however, was not increased by UG treatment in DEN-initiated mice (Table 2).

To determine whether hepatocyte proliferation rates differed between treatment groups, mice were treated with BrdU via osmotic pumps for 3 days before necropsy, and BrdU incorporation was evaluated immunohistochemically in liver sections. UG did not increase nonlesioned hepatocyte LI in initiation control or DEN-initiated mice (Table 3). The hepatocyte LI of AHF was substantially greater than that of nonlesioned liver, as expected (*27–29*). The mean hepatocyte LI of AHF from DEN/UG-treated mice was 29% greater ($p < 0.01$) than the mean hepatocyte LI of AHF from mice treated with DEN alone (Table 3). For these data, the hepatocyte LI of all the AHF in a given mouse were averaged together, and the value shown in Table 3 is the grand mean of all the mice in a group. Since different numbers of AHF were counted for each mouse, this value could be unduly influenced by mice with relatively few AHF. Thus, we also calculated the mean LI of the aggregate of UG-treated AHF (36.9 ± 7.6%, $n = 63$) and the aggregate of control AHF (28.7 ± 9.4%, $n = 99$), and the difference between the treatment groups remained highly significant ($p < 0.0001$).

Table 3. Hepatocyte BrdU labeling indices in lesioned and nonlesioned liver of male mice

	Hepatocyte BrdU labeling index (%)	
Treatment[a]	Lesioned[b]	Nonlesioned[c]
Saline/control	NA	0.37 ± 0.17
Saline/UG	NA	0.28 ± 0.12
DEN/control	29.7 ± 4.9	0.49 ± 0.30
DEN/UG	37.0 ± 4.0*	0.38 ± 0.24

Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; UG, unleaded gasoline; DEN, *N*-nitrosodiethylamine; NA, not applicable.
[a]Male B6C3F$_1$ mice were injected with DEN (5 mg/kg, intraperitoneally) at 12 days of age. Beginning at 5–7 weeks of age, mice were treated with 0 (control) or 2046 ppm UG vapor for 6 hr/day and 5 days/week for 16 weeks.
[b]Values are the means ± SD of the mean labeling indices of altered hepatic foci from mice.
[c]Values are the means ± SD of 8–9 mice.
*$p < 0.05$ as compared to DEN/control group.

Consistent with the increased proliferative rate of AHF from UG-treated mice, UG caused a shift in the size of AHF foci toward greater size relative to AHF from control mice (Fig. 1). This finding, which was obtained by counting the number of hepatocellular nuclei of AHF, independently corroborated the increase in mean size estimated by quantitative stereology (Table 2). However, the average hepatocyte LI of AHF in the different size classes was similar within a given treatment group [e.g., the proliferative rate in AHF of all size classes in the DEN/UG group was about 37% (data not shown)].

Discussion

Our data demonstrate that UG exposure under similar exposure conditions to the cancer bioassay of UG (*2*) increased the size of AHF as well as the number of macroscopic neoplasms in DEN-initiated male B6C3F$_1$ mice. Because UG did not also increase the number of AHF in DEN-initiated mice, the increased number of macroscopic neoplasms was likely a reflection of the stimulation of growth of AHF by UG. The failure of UG to increase the number of AHF in male or female mice (*5,11*) is consistent with the lack of significant genotoxic activity of UG (*7–9*). By standard definitions of tumor promotion (*30*), these data demonstrate that UG is a liver tumor promoter in male B6C3F$_1$ mice. UG also induced hepatic PROD

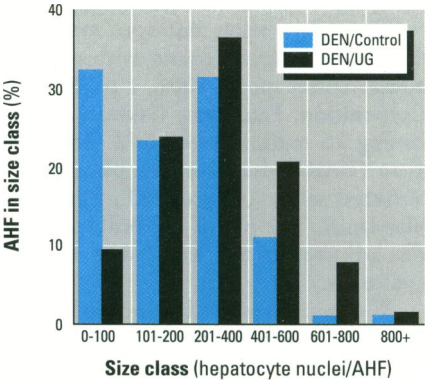

Figure 1. Size class of distribution of altered heptic foci (AHF) in *N*-nitrosodiethylamine (DEN)-initiated mice. Male B6C3F$_1$ mice were injected intraperitoneally with DEN (5 mg/kg) or saline. Beginning at 5–7 weeks of age, mice were exposed to 0 or 2046 ppm of unleaded gasoline (UG) vapor for 6 hr/day, 5 days/week, for 16 weeks. The number of cells in all AHF ≤0.85 mm in diameter was determined on 5'-bromodeoxyuridine-stained liver sections (n = 8–9), and the AHF were grouped into size classes as shown. The number of AHF in each size class is given as a percentage of the total counted, which was 99 and 63 in the DEN/control and DEN/UG groups, respectively.

activity, which fits with the correlation of CYP2B induction and the phenomenon of liver tumor promotion in rodents (*25,26*).

The liver-tumor-promoting activity of UG in male mice in this study appears to be at odds with the lack of carcinogenic activity in male mice in the cancer bioassay of UG (*2*). There are several potential reasons for this apparent discrepancy. First, the infant mouse initiation–promotion model used to detect the tumor promotion effect of UG may be more sensitive than the cancer bioassay, and only relatively robust promotional effects in the infant mouse model predict promotion activity in longer-term studies. For example, under identical exposure conditions, UG induced a fourfold increase in volume of AHF in female $B6C3F_1$ mice (*11*) versus a twofold increase in male mice in the present study, and UG induced liver tumors in female and not in male mice in the cancer bioassay (*2*). However, the quantitative difference in UG promotion activity between male and female mice most likely is, in part, a consequence of the 16-week time point chosen for analysis. AHF in the DEN/control group were about fourfold larger in male than in female mice at this time point (*11*), which is consistent with the well-established faster growth of AHF in male mice in the infant mouse model (*28,29*). Thus, it would have been relatively difficult to demonstrate the same fourfold increase in size of AHF in male versus female mice at the 16-week time point. Nevertheless, these data caution against the use of sensitive tumor promotion models to predict cancer bioassay outcomes.

A second possible explanation for why UG acted as a liver tumor promoter in the present study but was not hepatocarcinogenic in male mice in the chronic bioassay is that the biology of DEN-initiated foci may be significantly different from the biology of the spontaneous foci that UG presumably acted upon in the chronic bioassay. It is well known that the phenotypes and genotypes of AHF are heterogeneous, even among morphologically similar AHF (*17,31,32*). DEN is a potent mutagen, and its administration to mice yields a spectrum of AHF genotypes that may not be the same ones that progressed to liver tumors in the cancer bioassay of UG (*17,33*).

A third potential explanation for the apparent discrepancy between the present study and the results of the cancer bioassay of UG is that some factor(s) may be lacking in males, or present in females, that allows growth and progression of UG-promoted AHF to hepatic tumors selectively in females. For example, the particular hormonal milieu of the female mouse may have favored the progression of the types of spontaneous AHF promoted by UG in the cancer bioassay.

A novel finding in the present study was the small (~30%) but highly significant increase in hepatocyte LI in basophilic AHF from DEN-initiated, UG-treated mice relative to AHF from mice treated with DEN alone. UG did not increase the proliferative rate of surrounding nonlesioned hepatocytes, which is consistent with previous data showing that UG-induced hepatocyte proliferation disappeared after the first week of chronic, intermittent exposure (*4*). We are aware of only one other report (*34*) in which a mouse liver tumor promoter was shown to selectively increase the proliferative rate of AHF in male mice. In that study, phenobarbital selectively increased the LI of eosinophilic and not basophilic AHF but concomitantly suppressed LI in nonlesioned hepatocytes in female C3H mice (*34*). In rats, phenobarbital increased the number and size of DEN-initiated γ-glutamyl transpeptidase-positive nodules but did not change the average LI of these nodules (*32*). In another study, the LI of γ-glutamyl transpeptidase-positive hepatocytes as well as the LI of nonlesioned hepatocytes was increased by several liver tumor promoters, including phenobarbital, cyproterone acetate, and nafenopin (*27*).

The fact that AHF were larger, on average, in UG-treated mice might be a consequence of either a greater cellular birth rate, a decreased cellular death rate, or both, in these focal hepatocytes (*6*). The finding of a higher LI in AHF of UG-treated mice suggests that a greater cellular birth rate in these AHF at least contributed to promotion by UG. However, the molecular mechanism behind this increased proliferative rate remains to be determined. We proposed that UG might have antiestrogenic effects in female mice and that estrogen antagonism might secondarily lead to liver tumor promotion (*11,19*). The molecular basis for the inhibitory effect of estrogen on promotion in mice is not known but has been observed in both male and female mice (*13,14,18,19*). The antiestrogenic hypothesis seems less plausible in male mice, which would be expected to have lower endogenous levels of estrogen for the UG to act upon. Indeed, we did not detect any effect of UG on testes or pituitary of male mice, whereas UG decreased ovarian and pituitary weight in female mice (*11*). However, we cannot rule out the possibility that UG-induced liver tumor promotion in our two-stage model follows a similar antiestrogenic mechanism in both sexes of mice. Clearly, more studies are needed to define the biochemical/molecular mechanism of UG-induced tumor promotion in both sexes of mice.

It is noteworthy that wholly vaporized UG, which has the composition of liquid gasoline, was used in both the cancer bioassay of UG (*2*) and the present liver tumor promotion study of UG, whereas most human exposure to UG involves UG vapor. The low boiling-point components of liquid UG are disproportionately represented in UG vapor (*1,35*). However, high boiling-point components of UG, which account for the bulk of the hepatic mitogenic and P450-inducing activity of liquid UG, are present in wholly vaporized UG (*36*). Thus, if such hepatic activities are causally related to liver tumor promotion by UG, the results of this tumor promotion study and the cancer bioassay of UG may well overstate human liver cancer risk from UG exposure.

In summary, we have shown that UG promotes the development of DEN-initiated AHF in male mice under exposure conditions similar to those that failed to increase liver tumor incidence in a cancer bioassay of UG. Promotion of DEN-initiated AHF in male mice was associated with a selective increase in proliferation of focal hepatocytes. Further studies are needed to define the mechanism of growth stimulation of hepatic preneoplastic lesions by UG and elucidate the reason such stimulation results in hepatic neoplasia selectively in female mice. Our findings illustrate that tumor-promoting activity does not necessarily predict carcinogenicity and that analysis of chemically induced hepatocyte proliferation in nonlesioned liver does not necessarily predict induced proliferation in preneoplastic lesions.

References

1. Page NP, Mehlman M. Health effects of gasoline refueling vapors and measured exposures at service stations. Toxicol Ind Health 5:869–890 (1989).
2. MacFarland HN, Ulrich CE, Holdsworth CE, Kitchenv DN, Halliwell WH, Blum SC. A chronic inhalation study with unleaded gasoline vapor. J Am Coll Toxicol 3:231–248 (1984).
3. Magaw RI, Richter WR, MacGregor JA. A reexamination of liver tumors in mice exposed to wholly vaporized unleaded gasoline. J Am Coll Toxicol 12:195–199 (1993).
4. Tilbury L, Butterworth BE, Moss O, Goldsworthy TL. Hepatocyte cell proliferation in mice after inhalation exposure to unleaded gasoline vapor. J Toxicol Environ Health 38:293–307 (1993).
5. Standeven AM, Goldsworthy TL. Promotion of preneoplastic lesions and induction of CYP2B by unleaded gasoline vapor in female B6C3F1 mice. Carcinogenesis 14:2137–2141 (1993).
6. Butterworth BE, Popp JA, Conolly RB, Goldsworthy TL. Chemically induced cell proliferation in carcinogenesis. IARC scientific publication no. 116. Lyon:International Agency for Research on Cancer, 1992;279–305.
7. Conaway CC, Schreiner CA, Cragg ST. Mutagenic evaluation of petroleum hydrocar-

bons. In: Proceedings of the symposium: the toxicology of petroleum hydrocarbons (MacFarland HN, Holdsworth CE, MacGregor JA, Call RW, Kane ML, eds). Washington, DC:American Petroleum Institute, 1983; 128–138.
8. Loury DJ, Smith-Oliver T, Strom S, Jirtle R, Michalopoulous G, Butterworth BE. Assessment of unscheduled and replicative DNA synthesis in hepatocytes treated *in vivo* and *in vitro* with unleaded gasoline or 2,2,4-trimethylpentane. Toxicol Appl Pharmacol 85:11–23 (1986).
9. Richardson KA, Wilmer JL, Smith-Simpson D, Skopek TR. Assessment of the genotoxic potential of unleaded gasoline and 2,2,4-trimethylpentane in human lymphoblasts *in vitro*. Toxicol Appl Pharmacol 82:316–322 (1986).
10. MacGregor JA, Richter WR, Magaw RI. Uterine changes in female mice following lifetime inhalation of wholly vaporized unleaded gasoline: a possible relationship to liver tumors? J Am Coll Toxicol 12:119–126 (1993).
11. Standeven AM, Wolf DC, Goldsworthy TL. Interactive effects of unleaded gasoline and estrogen on liver tumor promotion in female B6C3F1 mice. Cancer Res 54:1198–1204 (1994).
12. Vesselinovitch SD, Mihailovich N. The effect of gonadectomy on the development of hepatomas induced by urethan. Cancer Res 27:1788–1791 (1967).
13. Rice TM. The biological behaviour of transplacentally induced tumours in mice. In: Transplacental Carcinogenesis (Tomatis L, Mohr U, Davis W, eds). Lyon:International Agency for Research on Cancer, 1973;71–83.
14. Lee G-H, Nomura K, Kitagawa T. Comparative study of diethylnitrosamine-initiated two-stage hepatocarcinogenesis in C3H, C57BL, and BALB mice promoted by various hepatopromoters. Carcinogenesis 10:2227–2230 (1989).
15. Goldfarb S, Pugh TD. Ovariectomy accelerates the growth of microscopic hepatocellular neoplasms in the mouse: possible association with whole body growth and fat deposition. Cancer Res 50:6779–6782 (1990).
16. Tsutsui S, Yamamoto R, Iishi H, Tatsuta M, Tsuji M, Terada N. Promoting effect of ovariectomy on hepatocellular tumorigenesis induced in mice by 3′-methyl-4-dimethylaminoazobenzene. Virchows Arch B Cell Pathol 62:371–375 (1992).
17. Hanigan MH, Winkler, ML, Drinkwater NR. Induction of three histochemically distinct populations of hepatic foci in C57BL/6J mice. Carcinogenesis 14:1035–1040 (1993).
18. Yamamoto R, Tatsuta M, Terada N. Suppression by oestrogen of hepatocellular tumourigenesis induced in mice by 3′-methyl-4-dimethylaminoazobenzene. Br J Cancer 68:303-307 (1993).
19. Standeven AM, Blazer DG III, Goldsworthy TL. Investigation of antiestrogenic properties of unleaded gasoline in female mice. Toxicol Appl Pharmacol 127:233–240 (1994).
20. Harada T, Maronpot RR, Morris RW, Stitzel KA, Boorman GA. Morphological and stereological characterization of hepatic foic of cellular alteration in control Fischer 344 rats. Toxicol Pathol 17:579–593 (1989).
21. Pugh TD, King JH, Koen H, Nychka D, Chover J, Wahba G, He Y-Z, Goldfarb S. Reliable stereological method for estimating the number of microscopic hepatocellular foci from their transections. Cancer Res 43:1261–1268 (1983).
22. Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T, Guengerich FP. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. Arch Biochem Biophys 238:43–48 (1985).
23. Nakajima T, Elovaara E, Park SS, Gelboin HV, Hietanen E, Vainio H. Monoclonal antibody-directed characterization of benzene, ethoxyresorufin and pentoxyresorufin metabolism in rat liver microsomes. Biochem Pharmacol 40:1255–1261 (1990).
24. Honkakoski P, Kojo A, Lang MA. Regulation of the mouse liver cytochrome P450 2B subfamily by sex hormones and phenobarbital. Biochem J 285:979–983 (1992).
25. Lubet RA, Nims RW, Ward JM, Rice JM, Diwan B. Induction of cytochrome P450b and its relationship to liver tumor promotion. J Am Coll Toxicol 8:259–268 (1989).
26. Jones CR, Lubet RA. Induction of a pleiotropic response by phenobarbital and related compounds: response in various inbred strains of rats, response in various species and the induction of aldehyde dehydrogenase in Copenhagen rats. Biochem Pharmacol 44:1651–1660 (1992).
27. Schulte-Hermann R, Schuppler J, Ohde G, Timmermann-Trosiener I. Effect of tumor promoters on proliferation of putative preneoplastic cells in rat liver. Carcinogenesis 7:99–104 (1982).
28. Goldfarb S, Pugh TD, Koen H, He Y-Z. Preneoplastic and neoplastic progression during hepatocarcinogenesis in mice injected with diethylnitrosamine in infancy. Environ Health Perspect 50:149–161 (1983).
29. Hanigan MH, Kemp CJ, Ginsler JJ, Drinkwater NR. Rapid growth of preneoplastic lesions in hepatocarcinogen-sensitive C3H/HeJ male mice relative to C57BL/6J male mice. Carcinogenesis 9:885–891 (1988).
30. Schulte-Hermann R. Tumor promotion in the liver. Arch Toxicol 57:147–158 (1985).
31. Dragan YP, Pitot HC. The role of the stages of initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat. Carcinogenesis 13:739–750 (1988).
32. Chen Z-Y, Farin F, Omiecinski CJ, Eaton DJ. Association between growth stimulation by phenobarbital and expression of cytochromes P450 1A1, 1A2, 2B1/2, and 3A1 in hepatic hyperplastic nodules in male F344 rats. Carcinogenesis 13:675–682 (1992).
33. Rumsby PC, Barrass NC, Phillimore HE, Evans JG. Analysis of the Ha-ras oncogene in C3H/He mouse liver tumors derived spontaneously or induced with diethylnitrosamine or phenobarbitone. Carcinogenesis 12:2331–2336 (1991).
34. Pereira MA. Comparison in C3H and C3B6F1 mice of the sensitivity to diethylnitrosamine-initiation and phenobarbital-promotion to the extent of cell proliferation. Carcinogenesis 14:299–302 (1993).
35. Wixtrom RN, Brown SL. Individual and population exposures to gasoline. J Exposure Anal Environ Epidemiol 2:23–78 (1992).
36. Standeven AM, Goldsworthy TL. Identification of hepatic mitogenic and cytochrome P-450-inducing fractions of unleaded gasoline in B6C3F1 mice. J Toxicol Environ Health 43:213–224 (1994).